Cancer Chemother Pharmacol (1993) 32: 249–254

Cancer Chemotherapy and Pharmacology

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The effect of NC-190, a novel antitumor compound, on the cell-cycle progression of HeLa S3 cells

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Received 22 September 1992/Accepted 17 February 1993

Abstract. A novel antitumor compound, *N*-β-dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxybenzo[a]phenazine-6-carboxamide sodium salt (NC-190), has potent antitumor activity against in vivo and in vitro tumor models. In this study, we evaluated the cell-cycle effect of NC-190 on cultured HeLa S3 cells using DNA/bromodeoxyuridine(BrdU) bivariate flow-cytometric analysis. Continuous treatment with NC-190 for 72 h inhibited the growth of cultured HeLa S3 cells in a concentration-dependent manner. Its 50% growth-inhibitory concentration (IC_{50}) was 0.039 μg/ml (0.085 μ*M*). The cell-cycle effects of NC-190 were dependent on the drug concentration and the treatment period. Continuous treatment with a low concentration (0.1 μg/ml) of NC-190 inhibited cell-cycle progression from the G_2 to the M phase, resulting in G_2 accumulation. With increasing concentration, NC-190 delayed cell-cycle traverse in the S and G_2 phases. At a higher (10 μg/ml) concentration of NC-190, cell-cycle traverse was prevented in the G_1, S, and G_2 phases. Under such conditions, NC-190 increased the numbers of S_0-phase cells (the cells with DNA content corresponding to that of S-phase cells, but without BrdU incorporation). Treatment for 2 h with NC-190 at 10 μg/ml induced the accumulation of cells in the G_2 phase, and cell debris was observed at 48 and 72 h after drug treatment. During this time, the proportion of cells in the S_0 phase increased up to 19.2%. The colcemid-induced mitotic cell accumulation was delayed by NC-190 at a concentration of 0.1 μg/ml at 4 h after the addition of the drugs. The addition of higher concentrations (1 and 10 μg/ml) of NC-190 inhibited the increase in the mitotic fraction completely. These results indicate that the mechanism involved in the G_2 arrest and the increment of S_0-phase cells caused by NC-190 is associated with this compound-induced cell death.

Abbreviations: IC_{50}, concentration necessary for 50% inhibition of cell growth; BrdU, bromodeoxyuridine; S_0 cells, BrdU-negative cells with the DNA content between G_1 and G_2M DNA contents

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Introduction

A novel antitumor compound, *N*-β-dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxybenzo[a]phenazine-6-carboxamide sodium salt (NC-190), has potent antitumor activity against in vivo and in vitro tumor models [6, 7, 9]. This agent is one of the most active compounds against P388 leukemia among the benzophenazine derivatives. NC-190 has stronger effects than does Adriamycin on P388 leukemia and Lewis lung carcinoma, and it has been found to have a significant antitumor effect on intraperitoneally implanted L1210 leukemia, B16 melanoma, M5076 reticulum-cell sarcoma, sarcoma 180 in mice, MH134 hepatoma, Yoshida sarcoma, and AH130 ascites hepatoma in rats, with optimal doses resulting in an increase in life span ranging from 98% to over 300% [6].

Previous studies by Tsuruo et al. [9] have shown that NC-190 is active against Adriamycin- and vincristine-resistant tumor cells in vivo and against pleiotropic drug-resistant tumor cells in vitro and that it inhibits the DNA topoisomerase II activity of the tumor cells.

Further studies [7] have shown that NC-190 has strong cytotoxic activity against several tumor cell lines in culture and that it inhibits DNA synthesis more than RNA and protein syntheses. Spectroscopic studies indicated that NC-190 directly interacted with calf-thymus DNA. Studies examining the colony formation of HeLa S3 cells indicated that NC-190 is an AUC-dependent and cell-cycle-phase–nonspecific cytotoxic agent. In the present study, we evaluated the effect of NC-190 on the cell-cycle progression of cultured HeLa S3 cells.

Materials and methods

Cell culture. HeLa S3 cervical carcinoma cells were purchased from Dainippon Pharmaceutical Co. Ltd. and were maintained in monolayer culture at 37° C in a humidified atmosphere of 5% CO_2/95% air in Eagle's minimal essential medium (MEM; Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Gibco) and gentamicin (80 μg/ml).

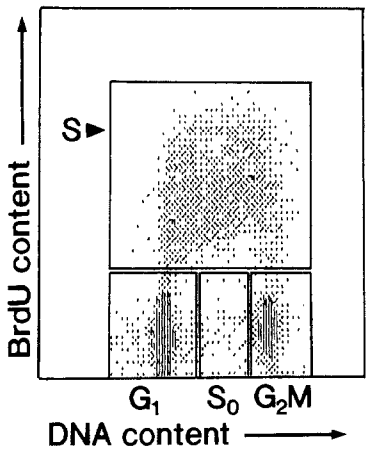

Fig. 1. Bivariate DNA/BrdU distribution of HeLa S3 cells. The distribution is shown for untreated control cells. The locations of cells in the G_1, S, S_0, and G_2M regions are indicated by each window

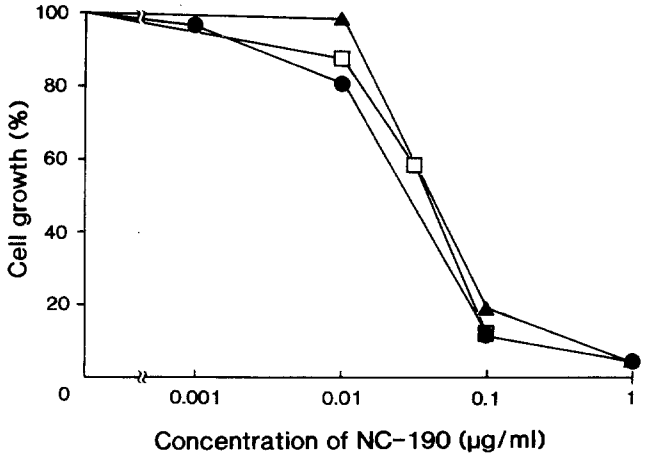

Fig. 2. The growth of HeLa S3 cells as a function of increasing concentrations of NC-190 over an incubation period of 72 h. The *curves* indicate different experiments

Drugs. *N*-β-Dimethylaminoethyl 9-carboxy-5-hydroxy-10-methoxybenzo[a]phenazine-6-carboxamide sodium salt (NC-190; molecular weight, 456.4) was synthesized in our laboratory. The NC-190 solution was prepared immediately prior to its use by dissolving the drug in dimethylsulfoxide. Control cultures recieved equivalent solvent (0.5%) exposure. Colcemid was purchased from Gibco and was dissolved in phosphate-buffered saline (PBS).

Quantitative measurements of the antiproliferative activity of NC-190. HeLa S3 cells were suspended in fresh growth medium at 2×10^4 cells/well in six-well plates (Falcon Plastics, Oxnard, Calif.) and incubated for 24 h at 37° C in a CO_2 incubator. Cells were then exposed to NC-190 for 72 h. After the treatment, the medium was removed and the cell layer was washed with PBS and trypsinized with an aliquot of 0.25% trypsin: ethylenediaminetetraacetic acid (EDTA; Gibco). PBS containing 2% fetal bovine serum was added to neutralize the trypsin. The cells were suspended by pipeting and enumerated with a ZM Coulter counter (Coulter Electronics Ltd., UK). The 50% growth-inhibitory concentration (IC_{50}) was calculated using the probit test.

Flow-cytometric analysis. Bivariate DNA/BrdU distributions were measured and analyzed by the methods of Dolbeare et al. [3]. HeLa S3 cells were continuously treated with NC-190 for various incubation periods. For the 2-h exposure experiment, NC-190 was washed off by rinsing the dishes twice with prewarmed MEM, with fresh medium being added and cells being further incubated at 37° C for the desired incubation periods. BrdU (Sigma) was added to the culture at 30 min before cell harvest at a final concentration of 10 μ*M*. The labeled cells were harvested and fixed in 70% ethanol. Fixed samples were stained with fluorescein isothiocyanate-labeled anti-BrdU antibody (Becton Dickinson Co.) and treated with propidium iodide (Sigma) to stain the DNA. Sample cells were analyzed by flow cytometry using an EPICS-753 flow cytometer (Coulter Electronics, Inc.). The argon-ion laser was tuned to the 488-nm line at a light power of 300 mW. Red fluorescence from propidium iodide was collected through a 630-nm-long band-pass filter and recorded as a measure of total DNA content. Green fluorescence from fluorescein was collected through a 525-nm band-pass filter and recorded as a measure of the amount of incorporated BrdU.

The data were analyzed by the modified method of Tsurusawa et al. [10] using gate windows. Four windows were set on the bivariate DNA/BrdU distribution to analyze cell-cycle progression (Fig. 1): (a) G_1, cells in the G_1 phase; (b) G_2M, cells in the G_2M phase; (c) S, BrdU-labeled cells; and (d) S_0, unlabeled cells with a DNA content between the G_1 and the G_2M DNA content [5]. Since we found that treatment with NC-190 increased the numbers of nonlabeled cells with a DNA content between that of G_1 cells and that of G_2 cells, we defined the S_0 cells as shown in Fig. 1.

Mitotic index. After the indicated incubation periods, cells were harvested and chromosome preparations were prepared by the standard procedures. Briefly, cells incubated in 75 m*M* potassium chloride for 20 ml in at 37° C were fixed with 3 : 1 (v/v) methanol-acetic acid. The fixative was changed three times, and aliquots of cell suspension transferred to slides were air-dried overnight and stained with Giemsa. The mitotic index was determined by scoring at least 2000 cells on a chromosome preparation.

Results

Growth-inhibitory activity of NC-190 on HeLa S3 cells

Figure 2 shows the growth-inhibitory effect of 72 h treatment with NC-190 on HeLa S3 cells. The growth of HeLa S3 cells was inhibited by NC-190 in a concentration-dependent manner. The IC_{50} value for NC-190 was 0.039 μg/ml (0.085 μ*M*, $n = 3$).

Continuous exposure of asynchronous HeLa S3 cells to NC-190

Exponentially growing HeLa S3 cells were exposed to different concentrations of NC-190 for 24 h. The changes in cell-cycle-phase distribution are shown in Fig. 3. Cells continuously incubated with NC-190 at 0.1 μg/ml demonstrated accumulation in the G_2M phase. The fraction of S-phase cells increased transiently, and the G_1 phase showed a steady decrease. At 24 h after the start of treatment, the fractions of S and G_2M cells declined slightly, with a corresponding increase being observed in the number of G_1-phase cells. This means that the G_2M-accumulated cells slowly progressed from the G_2M to the G_1 phase. Figures 4 and 5 show the cell-cycle distributions analyzed by DNA/BrdU bivariate analysis and the mitotic indices calculated after treatment with the indicated concentrations of NC-190 for 24 h. Low concentrations (0.1 and 0.3 μg/ml) of NC-190 produced a partial accumulation

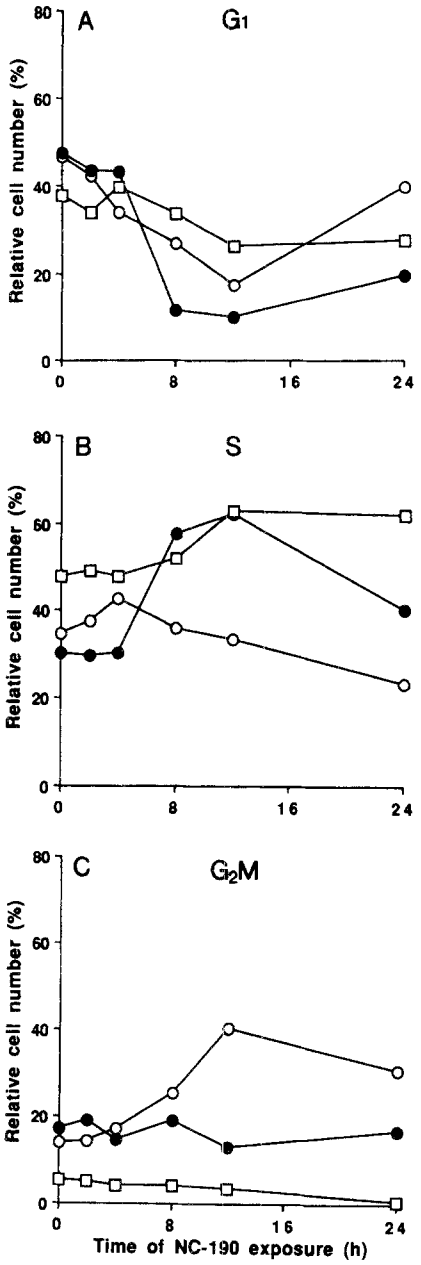

Fig. 3 A–C. Cell-cycle distribution of HeLa S3 cells sampled during continuous exposure to NC-190. DNA/BrdU distributions of HeLa S3 cells were analyzed. Cells were exposed to NC-190 at concentrations of 0.1 (○), 1 (●), and 10 μg/ml (□)

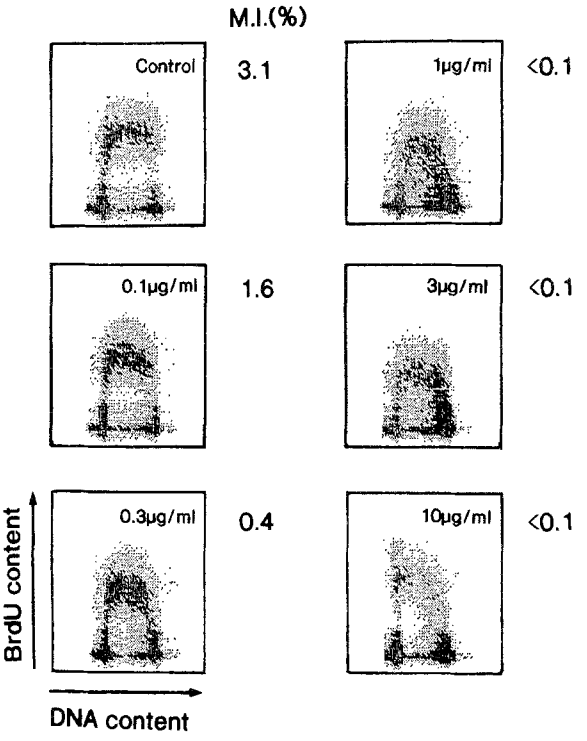

Fig. 4. Bivariate BrdU/DNA distributions and mitotic indices (*M. I.*) of HeLa S3 cells after treatment with NC-190 (0.1–10 μg/ml) for 24 h

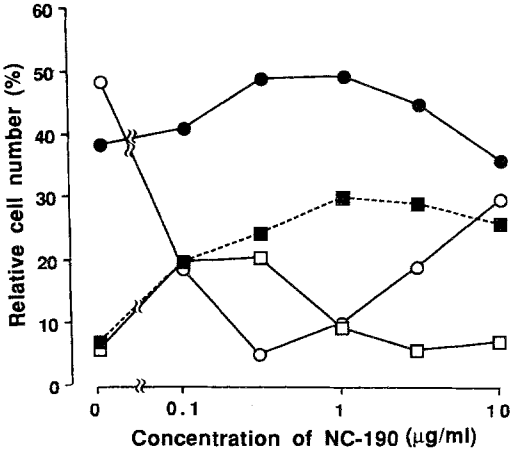

Fig. 5. Cell-cycle distribution of HeLa S3 cells exposed for 24 h to NC-190. ○, G_1; ●, S; □, G_2M; ■, S_0. DNA/BrdU distributions from Fig. 4 were analyzed

of G_2M-phase cells and decreased mitotic indices. This G_2M accumulation was primarily due to the accumulation of cells at the G_2 phase, since the mitotic indices of NC-190-treated cells were lower than the control value (Fig. 4). These results suggest that an NC-190 concentration of 0.1 μg/ml delayed the progression of cells from the G_2 phase to the M phase, resulting in partial G_2M accumulation.

With increasing concentration, NC-190 delayed cell-cycle traverse at the S phase. Continuous exposure to NC-190 at 1 μg/ml induced a marked increase in S-phase cells. The number of S-phase cells (30.1% at the start of treatment) reached 62.1% at 12 h after incubation. During this period, treatment with NC-190 resulted in a decrease in G_1-phase cells to 10.0% and in a constant G_2M compartment (Fig. 3). At high concentrations (3 and 10 μg/ml) of NC-190, the observed changes in cell-cycle-phase distribution were very small as compared with those seen in cells treated at 0.1 and 1 μg/ml (Figs. 3, 5). These results indicate that concentrations of NC-190 exceeding the range that produces G_2 arrest induced additional S-phase arrest and that even higher NC-190 concentrations also produced G_1 arrest.

We found that 24 h treatment with NC-190 induced a concentration-dependent increment of the S_0 cells

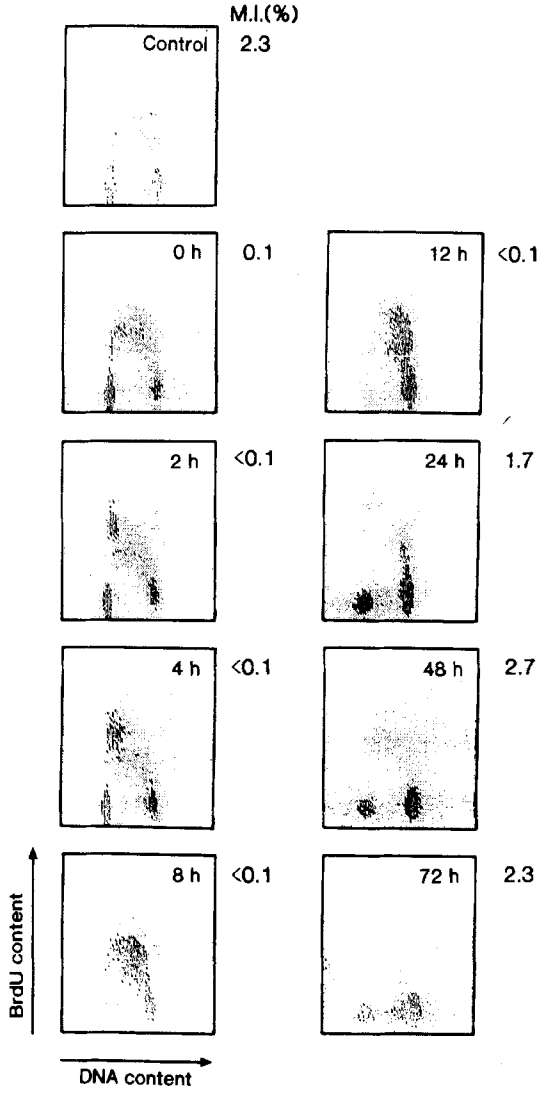

Fig. 6. Bivariate BrdU/DNA distributions and mitotic indices (*M. I.*) of NC-190-treated HeLa S3 cells. Following drug treatment (10 μg/ml for 2 h), cells were incubated in drug-free medium for the indicated intervals and then analyzed by flow cytometry

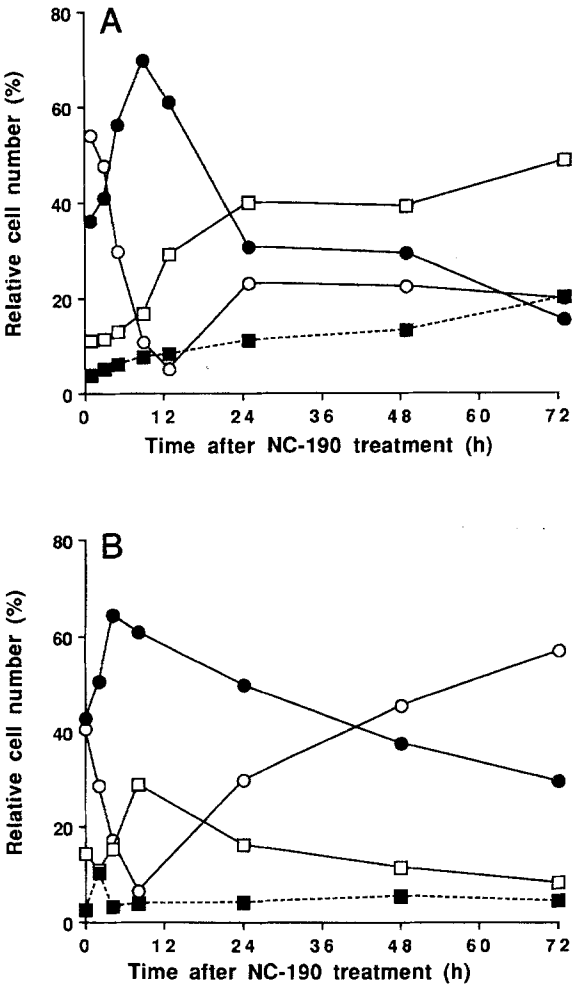

Fig. 7 A, B. Cell-cycle distribution of HeLa S3 cells after 2 h treatment with NC-190 at concentrations of 10 μg/ml (**A**) and 1 μg/ml (**B**). ○, G_1; ●, S; □, G_2M; ■, S_0

(Figs 4, 5). S_0 cells are cells that contain the corresponding DNA contents of the middle-S-phase cells and do not synthesize DNA. The treatment with NC-190 increased the number of S_0 cells (7.1% as a control value) up to 30.3% (1-μg/ml treatment, Fig. 5).

Treatment of HeLa S3 cells for 2 h with NC-190

Exponentially growing HeLa S3 cells were exposed to NC-190 at a concentration of 10 μg/ml for 2 h. The cells were washed twice with prewarmed MEM and fed with fresh medium. At the indicated times after drug treatment, samples were taken for flow cytometry. Figure 6 shows the DNA/BrdU distributions and mitotic indices, and the quantitative analyses of these DNA/BrdU distributions are shown in Fig. 7 A.

Treatment with NC-190 (10 μg/ml) resulted in a transient accumulation of cells in the S phase accompanied by a decline in the G_1 phase and a constant G_2M compartment. The mitotic index rapidly decreased to below 0.1% after the treatment. After 12 h, the cells progressed from the S to the G_2 phase, and the G_2 fraction increased to 47.8% (78 h). Although a recovery of the mitotic index was observed beginning at 24 h, the cell number did not increase (data not shown). This slight increase in the percentage of mitotic cells resulted in an increase in the number of G_1-phase cells but did not induce a return to the control value. These results indicate that this NC-190 treatment initially induced S and G_2 arrest and subsequently produced only G_2 arrest.

After 48 and 72 h, the bivariate DNA/BrdU distribution showed the formation of cell debris and hypertetraploid cells (Fig. 6), and the cell number decreased (data not shown). The S_0 fraction gradually increased, even after NC-190 had been removed, and reached 19.2% at 72 h after drug removal.

In the case of treatment with NC-190 at 1 μg/ml for 2 h, the changes in cell-cycle distribution were reversible and partial G_2 arrest was observed (Fig. 7B). The reversed cells grew to confluence, since the proportion of G_1 cells

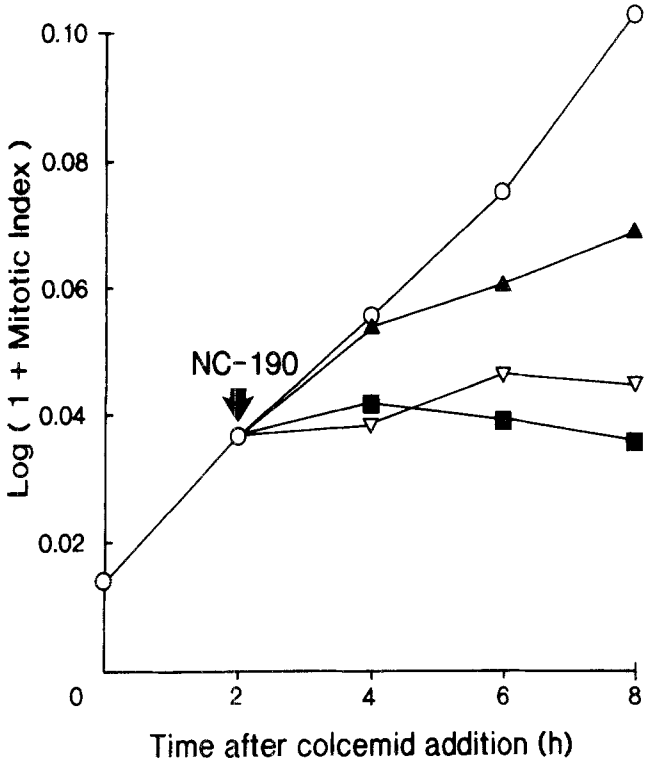

Fig. 8. Effect of NC-190 on mitotic cell accumulation. Exponentially growing HeLa S3 cells were exposed initially to colcemid (0.05 μg/ml) alone for 2 h, followed by the addition of NC-190 at concentrations of 0 (○), 0.1 (▲), 1 (▽), and 10 μg/ml (■) for 6 h

increased to about 60%. At this time, S_0 fractions did not increase.

Effect of NC-190 on mitotic cell accumulation

Exponentially growing HeLa S3 cells were treated with colcemid at 0.05 μg/ml, resulting in an incubation-time-dependent increase in mitotic cells (Fig. 8). At 2 h after the initiation of colcemid treatment, the indicated concentrations of NC-190 were added to the medium. The colcemid-induced mitotic cell accumulation was delayed by treatment with NC-190 at 0.1 μg/ml at 4 h after the addition of the latter. The addition of higher concentrations (1 and 10 μg/ml) inhibited the increase in the mitotic fraction of colcemid-treated cells. These results indicate that cell progression from the G_2 to the M phase was blocked immediately after the addition of NC-190.

Discussion

In this study, we examined the mode of action of NC-190 in connection with cell-cycle progression using the flow-cytometric technique. The cell-cycle effects of NC-190 were dependent on the drug concentration and the duration of treatment. Low concentrations (0.1 μg/ml) of NC-190 inhibited cell-cycle progression from the G_2 to the M phase, resulting in G_2 accumulation. In the case of a short (2-h) exposure, NC-190 at 10 μg/ml induced an accumulation of cells in the G_2 phase after their recovery from S-phase arrest. In colcemid-induced mitotic accumulation, NC-190 rapidly inhibited the increment of mitotic cells. From the results reported above, it may be concluded that the transition from G_2 to M occurs at the time when the cells studied are most sensitive to treatment with NC-190.

Tsuruo et al. [9] and Andoh et al. [1] have reported that NC-190 inhibits the activity of DNA topoisomerase II. The topoisomerase II-inhibitory activity of NC-190 may also contribute to the G_2 arrest of cells, since various topoisomerase II inhibitors induce G_2 arrest [2, 4]. Topoisomerase II has an important function in changing the topological structure of DNA, which is involved in the process of DNA replication, strand segregation, and chromatin condensation [11, 12]. Zucker et al. [13] have suggested that treatment with topoisomerase II inhibitors induces the formation of polyploid cells, which may be involved in the inhibition of chromosome condensation. After 2 h treatment with NC-190 at 10 μg/ml, DNA/BrdU bivariate analysis showed that this treatment generated hypertetraploid cells (Fig. 6). At this time, the endoreduplication was observed in chromatin preparations (data not shown). This observation suggests that part of the cells arrested in the G_2 phase following treatment with NC-190 subsequently progressed into the $>G_2$ polyploid stage.

In this study, we employed DNA/BrdU bivariate flow-cytometric analysis. This procedure enables a distinction to be made between DNA-synthesizing (S-phase) cells and DNA-nonsynthesizing cells with a S-phase DNA content (S_0 cells). We found that treatment with NC-190 increased the fraction of S_0 cells. Continuous exposure to NC-190 for 24 h increased the fraction of S_0 cells to 30.3% (1 μg/ml). In the case of the 2-h exposure experiment, the ratio of S_0 cells was increased to 19.2% at 72 h after treatment with NC-190 at 10 μg/ml.

Previously, we reported that treatment with an NC-190 concentration of 10 μg/ml for 2 h, which induced 99% inhibition in a colony-forming assay, sharply decreased the viability of HeLa S3 cells beginning at 48 h after drug treatment, and cell viability did not recover, even on day 13 [7]. In the present study, we also demonstrated that the same treatment with NC-190 resulted in increments of G_2- and S_0-phase cells beginning at 12 h after treatment and in the induction of cell debris and hypertetraploid cells starting at 48 h. These results indicate that the mechanism involved in the G_2 arrest and the increment of S_0-phase cells caused by NC-190 is associated with this compound-induced cell death.

In chick embryos, Tone et al. [8] reported that new cell populations, which did not belong to any phase of the normal cell cycle, appeared during the process of programmed cell death. The DNA content of one of the new cell populations (D_1) varied from 2n to 4n, but no DNA synthesis took place. Tone et al. suggest that this alteration in DNA synthesis seems to precede an actual manifestation of microscopically detectable changes directly leading to cell death. The D_1 population may be the same as the S_0 cells described in the present report. NC-190-induced increment of S_0 cells may be involved in the process of NC-190-induced cell death.

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